EXHIBIT A

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Epithelial Human Chorionic Gonadotropin Is Expressed and Produced in Human Secretory Endometrium During the Normal Menstrual Cycle[1]

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ABSTRACT

The objective of this study was to determine whether beta human chorionic gonadotropin (hCG) (*CGB*) subunits and alpha hCG (*CGA*) subunits are expressed and the hCG dimer is produced in normal human cyclic endometrium. Endometrial specimens were collected for histological dating from women undergoing treatment in our division of human reproduction. RNA from normal secretory endometrium was extracted, and *CGB* and *CGA* gene expression was assessed by semiquantitative PCR. Adequate secretory endometrial specimens were homogenized using protease inhibitors. Proteins present in the supernatant were separated electrophoretically, and molecular hCG isoforms were detected by Western blot. The supernatant hCG concentrations were measured by ELISA. We characterized hCG and leukocytes in endometrial specimens by immunohistochemistry. Uterine flushing was performed to confirm endometrial hCG secretion into the uterine fluid. A full-length *CGB* mRNA encompassing the exon 1 promoter region and the structure exons 2 and 3 (including the C-terminal peptide) was expressed in normal secretory endometrial specimens (similar to *CGA*) during the early secretory phase of the menstrual cycle, up to an optimum at the midsecretory to late secretory phases. In homogenate supernatants obtained from normal secretory endometrium, hormone concentrations of dimeric hCG were approximately 5 mU per 10 mg of tissue, compared with considerably smaller concentrations of corresponding single free *CGB* subunit. Single chains of *CGB*, *CGA*, and dimeric molecular hCG isoforms were found in endometrial specimens by Western blot. Glandular endometrial hCG production is demonstrated immunohistochemically, with an increase toward the late secretory phase vs. the early secretory phase of the normal secretory menstrual cycle. However, glandular hCG release is diminished or absent in the dyssynchronous or missing endometrial secretory transformation. Endogenous endometrial hCG may be important for implantation and maintenance of pregnancy.

human chorionic gonadotropin, human endometrium, implantation, mechanisms of hormone action, menstrual cycle

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INTRODUCTION

The human endometrium undergoes dramatic morphological and functional changes during the menstrual cycle. During the first half of the menstrual cycle, the estrogen-dominant proliferative phase is characterized by intense mitotic activity of glandular and stromal cells, and the second half (or progesterone dominant) secretory phase is noted by functional differentiation of the cellular components. Progesterone induces the onset of endometrial secretory transformation. In the human endometrium, stromal cells begin to visibly differentiate into decidua during the midsecretory phase of the normal menstrual cycle, even in the absence of implantation. Stromal cell differentiation begins around the spiral arteries and spreads through the upper compartment of the spongy endometrium site [1]. During the process of decidualization, synchronous stromal and glandular differentiation results in secretory activity of different hormones, growth factors, and eicosanoids [2–4]. Predecidualization of the human secretory endometrium is essential for embryo implantation and the maintenance of pregnancy. In this study, we hypothesized that human secretory endometrium is capable of synthesizing human chorionic gonadotropin (hCG).

Human chorionic gonadotropin is regarded as a pregnancy-specific hormone and is produced in the trophoblast. It is released in large quantities from the villous syncytiotrophoblast into the maternal blood. The heterodimeric glycoprotein hormone is composed of two noncovalently associated alpha and beta subunits. The common alpha hCG (*CGA*) subunit is encoded by a single alpha gene on chromosome 6. The beta hCG (*CGB*) subunit is encoded by a gene cluster of six homologous genes on chromosome 19 and by one of them as two allelic pseudogenes [5–7]. The *CGB* molecule is characterized by a specific *N*-linked glycosylation pattern and by an *O*-linked pattern in C-terminal peptide [CTP]) [8, 9]. The free *CGA* and *CGB* proteins combined form intact biologically active hCG. Human chorionic gonadotropin bioactivity is dependent on glycosylation, the structure of which changes during early pregnancy trophoblast production [10–12]. In previous studies [13–16], hCG production has been confirmed as a common phenomenon associated with several different types of carcinomas. Some authors have reported that *CGB* or hCG is also produced at low levels by various normal human healthy tissues, particularly in the intestinal, urinary, and respiratory tracts [17–20] and in the fallopian tubes [21]. Human chorionic gonadotropin/luteinizing hormone (LH) receptors have been described in the endometrium [22, 23] and were identified in several gonadal and nongonadal tissues [24].

In initial immunohistochemical and in situ hybridization examinations, we showed that glandular cells of the endometrium express and produce *CGB* subunit protein primarily during the secretory phase in the uterine epithelium [25, 26]. The objective of the present study was to investigate total *CGB*

and *CGA* gene expression, as well as hCG production, in endometrial tissue specimens exhibiting normal or abnormal proliferation and secretory phases during the menstrual cycle.

MATERIALS AND METHODS

Tissue Collection and Processing

To examine human *CGA* and *CGB* gene expression, endometrial tissues were obtained from 581 patients from the previous years who had undergone routine infertility investigations for this study. Endometrial specimens were collected after cervix dilatation and curettage of the uterine cavum for fertility evaluation and molecular biological examinations. In addition, endometrial samples from fertile patients who underwent hysterectomy for benign gynecological conditions other than endometrial disease were included. All biopsy specimens were obtained after informed consent from the patient, and the study was approved by the Medical Ethics Committee of the University of Leipzig.

Each tissue sample included was routinely staged by histological dating using criteria for the normal menstrual cycle [27], confirmed by independent histological examinations by an experienced pathologist. Endometrial specimens were collected from both the proliferative and secretory phases of the menstrual cycle and were divided into two groups. Samples from the first patient group demonstrated normal secretory endometrium from couples experiencing infertility solely owing to tubal damage, male factor, or unexplained infertility. Specimens were included in the study only if the clinical analysis suggested the absence of other gynecological pathologies. Women who had received any form of exogenous hormones or had used an intrauterine contraceptive device during the previous 3 mo were excluded. Endometrial samples in this group were categorized into the following five subgroups: proliferative (n = 60), early secretory (n = 42), midsecretory (n = 35), late secretory (n = 30), and late predecidual secretory (n = 38) endometrium. The remaining endometrial specimens were included in a second patient group that demonstrated dyssynchronous or missing secretory transformation and were used for comparative immunohistochemical and histological characterization with regard to the first patient group.

The collected endometrial samples were rinsed with saline to remove blood and were divided into aliquots. For histochemical and immunohistochemical evaluation, the tissues were immediately fixed in 4% neutral buffered formalin overnight and then embedded in paraffin. For total RNA extraction and subsequent semiquantitative RT-PCR and restriction enzyme analysis, samples were immediately submerged in RNA stabilization reagent (RNAlater; Qiagen) and then rapidly frozen and stored at −80°C for tissue homogenization. Western blot, and hormone detection, endometrial specimens were snap frozen and stored at −80°C until processed.

Endometrial Tissue Homogenization, Supernatant Preparation, and hCG Hormone Determination

Tissue specimens of approximately 30 mg were rinsed with PBS and resuspended in 300 µl of ice-cold 50 mM Tris-HCl buffer (pH 7.6) containing 200 mM NaCl (Tris-buffered saline (TBS)) with 0.5% sodium deoxycholate, 2 mM edetic acid, 1.0% Nonidet P-40, and one 10-ml Complete Protease Inhibitor Tablet Pre (Roche), 1 µmol/L of pepstatin; and inhibiting acid proteases. They were then disrupted using the Ultra-Turrax (IKA Works, Inc.) homogenization method on ice for 1 min. The postnuclear endometrial supernatant was prepared by repeated centrifugation of the homogenates at 15 000 × g and 4°C for 30 min and was immediately frozen at −20°C until examination. Hormone concentrations in endometrial homogenate supernatants (total hCG/*CGB*, free *CGB*, and LH) were determined using routine hormone test kits manufactured by Bayer (Advia Centaur total hCG/beta hCG immunoassay with two hCG antibodies having different epitope-binding capacities for the dimeric and free beta subunits of hCG, sensitivity of less than 2.0 mU/ml, and negligible cross-reactivity with LH and follicle-stimulating hormone [FSH]; and Advia Centaur LH immunoassay with two LH antibodies having different epitope-binding capacities, sensitivity of 0.07 mU/ml, and negligible cross-reactivity with hCG and FSH) and by Brahms (Kryptor free beta hCG ethanol immunoassay with two beta hCG antibodies and a sensitivity of 0.1 mU/ml), all with an intraassay precision of less than 3%.

Uterine Flushing and Hormone Determination

Uterine flushing in selected patients with subfertility was performed in the secretory phase during the implantation window on the day before curettage. The procedure was performed using a sterile bivalve speculum to expose the cervix, which was thoroughly cleaned with sterile saline (0.154 M sodium chloride). Two insemination catheters were passed into the uterine cavity through the cervix for continuous infusion of sterile normal saline at a low rate of 20 ml/60 min flow for 1 h and discontinuous collection of six fractionated uterine flushing fluid aliquots at successive 10-min intervals. The fraction volumes were detected, and the respective gonadotropin and steroid hormone levels were measured using hormone test kits from Roche (Roche Elecsys hCG plus beta immunoassay with two different hCG antibodies, sensitivity of 0.1 mU/ml, and cross-reactivity with LH and FSH of <0.1%; and Roche Elecsys LH immunoassay with two antibodies, sensitivity of 0.1 mU/ml, and cross-reactivity with other gonadotropins of <0.1% [similar to the Roche Elecsys FSH immunoassay]).

Isolation of RNA and RT-PCR

Total RNA was isolated from endometrial tissue and the early pregnancy placenta as a control. Approximately 30 mg of prewashed fresh or frozen samples stored at −80°C was disrupted and homogenized using an Ultra-Turrax. The homogenates were kept on ice for 5 min to ensure complete dissociation of the nucleoproteosomes. Total RNA was extracted using Trizol reagent (Gibco) according to the manufacturer's instructions. The pellets were dissolved in diethyl pyrocarbonate-treated water, and RNA samples of 30 µl were quantified and stored as small aliquots at −80°C.

Total RNA (2 µg) was treated with RNase-free DNase (Roche) and reverse transcribed to synthesize cDNA. The 2.5-µl aliquot of RNA was heated at 65°C for 5 min to eliminate the DNase and unfold the RNA, followed by cooling at 4°C. A 2.5-µl aliquot of cDNA reaction mixture was added to obtain a final concentration of 10 mM Tris-HCl (pH 8.30); 50 mM potassium chloride; 5 mM magnesium chloride; 1 mM each of deoxyadenosine triphosphate, 2-deoxycytidine-5-triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate (deoxyribonucleotide triphosphates [dNTP]); 10 IU/µl of RNase inhibitor; 12.5 IU/µl of avian myeloblastosis virus RT; and 0.2 µg/µl of oligodeoxythymidine primer (pdT15; Roche) in a final volume of 5 µl. The reaction was carried out using a Perkin-Elmer thermocycler and was performed using the following conditions: 25°C for 10 min and 42°C for 60 min, followed by a 5-min step at 94°C to destroy RT activity. All RT reaction reagents were obtained from Roche.

The PCR procedure was performed immediately after RT in the same tube by adding 20 µl of PCR mixture to the 5-µl RT reaction volume, resulting in a final concentration of 10 mM Tris-HCl (pH 8.30), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.2 mM of each dNTP, 1.25 IU/25 µl of AmpliTaq-Fast start (FS) DNA polymerase, and 10 pM/25 µl of each primer pair for the different *CGB*, *CGA*, and *GAPDH* oligonucleotides. All PCR amplifications included an initial denaturation step of 5 min at 95°C and a final elongation step of 10 min at 72°C. As summarized in Table 1, amplification of cDNA was performed using four different primer pairs specific to the respective *CGB* subunit, which resulted in amplicons of 548 bp [6], 423 bp [28], and 378 bp and 300 bp [29], as well as using primer pairs to *CGA* [30] and *GAPDH* [31]. All mRNA sequences expressed from genes 3, 5, 6, 7, and 8 of *CBG* were included in the amplification using the selected primer pairs. The PCR procedure was performed follows: 35 cycles of 30 sec at 94°C, 30 sec at 62°C, and 60 sec at 72°C for all primer pairs, excluding the 548-bp *CGB* amplification, for which the annealing time was 45 sec. This fragment size was specific to *CGB* RNA because the primers were located in different exons. To exclude the possibility of the amplification contaminating genomic DNA, despite DNase treatment, PCR was also performed excluding RT from the cDNA step for each primer set, and no positive PCR products were observed. A negative control reaction in which no RNA or cDNA template was added to the reaction mixture was included in each experiment. Placental RNA was applied to each primer set amplification as a positive control. All PCR amplification reagents were obtained from Roche.

All oligonucleotide primer pairs were synthesized (Applied Biosystems). Nine-microliter aliquots of the PCR products were electrophoresed in a 2.0% agarose gel in 50 mM Tris-buffered 150 mM saline buffer, pH 7.40, to prove the efficiency and fidelity of the *CGB*- and *CGA*-DNA fragment amplification, and 0.01% ethidium bromide was used for UV identification and documentation.

Restriction Enzyme Analysis

The identity of the 300-bp PCR product was verified by restriction enzyme digestion. Several *CGB*-specific restriction enzymes (*Styl*, *Bsp*1286, *HaeIII*, *AvaII*, and *SmaI*; Roche) were included in the cleavage experiments to confirm the PCR-derived DNA sequence of *CGB*, as opposed to that of beta LH (*LHB*) subunit. The enzyme concentrations and optimal buffer conditions for DNA digestion were selected according to the manufacturer's instructions. The RT-PCR products were separated electrophoretically in an agarose gel, and the 300-bp *CGB* cDNA amplicon was extracted using a DNA gel extraction kit

TABLE 1. Oligonucleotide primer pairs used for *CGB*, *CGA*, and *GAPDH* semi-quantitative reverse transcription reaction and PCR amplification procedure.[a]

No.	Gene	Primer location	Exon	Strand	Nucleotide sequence	Amplicon bp	Paired no.
1	*CGB*	−353/−	1	Sense	5′-TCGGGCCACGGCCTCCT-3′	548	4
2	*CGB*	−226/−	1	Sense	5′-TCACTTCACCGTGGTCTCCG-3′	423	4
3	*CGB*	108/127	2	Sense	5′-GGCTGTGGAGAAGGAGGGCT-3′		5,6
4	*CGB*	197/178	2,3	Antisense	5′-CAGCACGCGGGTCATGGT-3′		1,2
5	*CGB*	406/384	3	Antisense	5′-CGAACGCGGGGTCATCACAGGTC-3′	300	3
6	*CGB*	484/463	3	Antisense	5′-TCGGGGTGTCTCGCAGGCC-3′	370	3
7	*CGA*	83/102		Sense	5′-GCAGGATTGCCCAGAATGC-3′	231	8
8	*CGA*	313/294		Antisense	5′-CCGTGTGGTTCTCCACTTTG-3′		7
9	*GAPDH*	325/352		Sense	5′-CCATGGAGAAGGCTGGGG-3′	196	10
10	*GAPDH*	530/510		Antisense	5′-CCAAAGTTGTCATGGATGACC-3′		9

[a] The specific *CGB* primer does not recognize *LHB* expression.

(Qiagen). DNA digestion fragments obtained after overnight incubation at 37°C were visualized in a 2.0% agarose gel. The patterns of the cleavage products obtained by digestion of the 300-bp amplicon with *Xcl* (300 bp), *Bpi*1285 (175/125 bp), *Hae*III (300 bp), *Ava*II (94/87/43/33 bp), and *Sma*I (94/87/43/43/33 bp) should have been consistent with the *LHB* DNA sequence.

Immunohistochemistry

The divided sample parts of fresh endometrial tissue collected from the curettage specimen were used in parallel for PCR studies and immunohistochemical staining. The tissue samples were fixed in 4% phosphate-buffered 4% paraformaldehyde overnight and embedded in paraffin. The tissue blocks of endometrium and paraffin blocks of early pregnancy placenta as a control were cut into 4-µm-thick serial tissue sections, mounted on superfrost slides, deparaffinized, cleared in xylene, rehydrated in a series of ethanol, and incubated for 10 min in 50 mM TBS with 0.1% Tween-20 [pH 7.60] (TBST). After rehydration, the sections of the specimens were incubated with 0.3% fresh hydrogen peroxide in methanol (30 min) to block endogenous peroxidase activity.

Immunolocalization of hCG. Immunohistochemical staining for hCG was first performed using polyclonal antibody. After a brief wash in TBS, the tissue sections were placed in a humidified chamber and sequentially overlaid and incubated with 100 µl of each the following reagents at room temperature: 1) TBS with 0.2% Triton X-100 for 10 min for antigen demasking; 2) avidin and biotin blocking solution (DAKO) for 10 min each for endogenous biotin suppression; 3) 10% normal goat serum (NGS) in TBS for 30 min to block nonspecific staining; 4) after removal of excess NGS blocking serum, general hCG staining with primary rabbit anti-*CGB* (A0231; DAKO) (diluted 1:500 in TBST/10% NGS) or with primary rabbit anti-CGB-CTP (Biotrend) (diluted 1:500 in TBST/10% NGS) at 4°C overnight; 5) using the Elite ABC kit (Vector), biotinylated goat anti-rabbit IgG as secondary antibody diluted 1:2000 in TBST/NGS for 30 min; 6) peroxidase (POD)-conjugated avidin-biotin complex (Vectastain ABC; Vector) for 30 min; and 7) diaminobenzidine (DAB) from Vector for 5 min to develop the brown reaction product, between each reagent step performed in the humidified chamber, the sections were rinsed three times for 5 min with TBS. Negative controls consisted of samples for which the primary hCG or other primary antibodies were omitted from the TBST/NGS solution. A positive control section of early pregnancy placental tissue was included for the primary antibodies used in every staining protocol as described for endometrial tissues. The specimens were initially mounted on aqueous-based Histogel from Lhurin and were later dehydrated, cleared in xylene, and mounted with nonaqueous permanent mounting medium. Human chorionic gonadotropin was then detected in endometrial sections using different mouse monoclonal antibodies specific for *CGB* and *CGA* subunit epitopes. For hCG staining with mouse anti-*CGB* antibodies (INN-2 or INN-22 diluted 1:5000 or 1:100, Serotec) or with mouse anti-*CGA* antibody (INN-132 diluted 1:200 [Serotec] in TBS/normal rabbit serum [NRS]), the Catalyzed Signal Amplification (CSA) system (DAKO) was used according to the manufacturer's instructions. After treatment with Target retrieval solution (pH 6.2; DAKO) for 20 min at 95°C, endogenous POD blocking of endometrium sections with 0.3% hydrogen peroxide in methanol, avidin-biotin blocking, and blocking of nonspecific antibody binding site with TBST/10% NRS, incubation with the primary antibodies was continued at 4°C overnight, followed by incubation with secondary biotinylated rabbit anti-mouse IgG antibody diluted 1:2000 in TBS/NRS and with POD-conjugated avidin-biotin complex (DAKO), each for 30 min. A supplementary amplification step was included in the CSA system to increase biotin signals localized at the antibody binding site. Finally, incubation with the POD-avidin conjugate allowed for amplification of the DAB staining reaction.

Immunolocalization of endometrial leukocytes and endothelial cells. Immunohistochemical staining of endometrial mononuclear cells was performed using monoclonal mouse antibody against the leukocyte common antigen CD45 (clone 2B11 and PD7/26) from DAKO. After blocking of endogenous peroxidase, avidin-biotin, and nonspecific antibody binding as already described, the primary antibody (1:50) was incubated with tissue sections for 1 h at room temperature, followed by treatment with biotinylated secondary rabbit anti-mouse IgG antibody and POD-conjugated avidin-biotin complex according to instructions included in the Vectastain ABC kit. Peroxidase activity was visualized by incubating the samples with DAB for 5 min. Counterstaining was performed with hematoxylin.

To examine natural killer (NK) cells and vascularization of the endometrium, a sequential double immunostaining protocol was used for serial tissue sections. Immunostaining was performed using a monoclonal human anti-CD56 neural cell adhesion molecule (NCAM) NK cell primary antibody (Santa Cruz), followed by a monoclonal anti-CD34 antibody (clone QBEND/ 10; Serotec) after development of the first chromogen. CD56 antigen staining required demasking pretreatment of sections with Target retrieval solution (pH 6.2) for 20 min at 95°C in a water bath, followed by 20 min of cooling. After the already described blocking steps, the normal rabbit serum-preincubated tissue sections were treated with anti-CD56 (diluted 1:100) at 4°C overnight. This was followed by the CSA system procedure using secondary biotinylated rabbit anti-mouse mouse IgG antibody, POD-conjugated avidin-biotin complex, and DAB chromogen visualization as already described. After brief washing, the sections were treated with the anti-CD34 antibody (an endothelial cell marker) diluted 1:50 for 1 h at RT, followed by alkaline phosphatase-conjugated avidin-biotin complex using the Vectastain AB-AP kit (Vector) with AP blue chromogen for CD34 visualization.

Characterization of Endometrial Secretory Transformation

As demonstrated in the present study, we believe that the degree (range, 0–4) of immunohistochemical hCG staining of endometrial glands characterizes the dating of normal, diminished, or failing uterine secretory transformation with respect to the cycle phase-adequate glandular shape configuration. Lack of hCG staining (hCG, 0) represents the proliferative phase, low staining (hCG, 1–2) represents the early secretory phase, higher staining (hCG, 2–3) represents the midsecretory phase, and strong staining (hCG, 3–4) represents the late secretory phase of normal endometrial transformation. Decreased or zero hCG staining relative to the cycle phase-respective values reflects diminished or failing secretory transformation of the endometrium.

In general, the eight histological dating criteria by Noyes et al. [27] are used in clinical practice for evaluation of cycle day-synchronous or delayed endometrial secretory transformation. Based on histological data from endometrium specimens in both of our patient groups, we believe it is more likely that the cause of infertility may be dyssynchronous endometrial secretory transformation between normal glandular shape configuration and delayed glandular nucleus differentiation and/or leukocyte proliferation or infiltration. Relative to conventional grading parameters of endometrial secretory transformation [32, 33], we morphologically characterized specimens using the following four endometrial criteria (score range, 1–6 [5 is optimal]) to compare and supplement the recommended hCG staining: A) glandular shape configuration (small and round; small, elongated, narrow lumen; elongated, tortuous, coiled; dilated, round, expanded; dilated, papillar sinus, beginning of serration; saw tooth-like), B) glandular epithelial nucleus configuration and localization (polystratified; tall columnar, cylindrical, basal located; subnuclear vacuoles; longish, rounded; oval; round), C) stromal cell density and configuration (undifferentiated, dense; spindle shaped, broken up; rounded cells, dense; rounded cells, broken up; edematous; predecidual), and D)

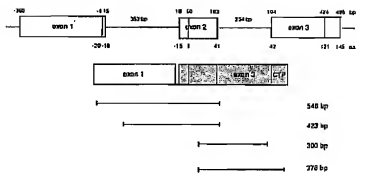

FIG. 1. *CGB* gene expression in human secretory endometrium. A) The gene sequence shows the extent of the exon 1 promoter region and structure exons 2 and 3 present in the *CGB* subunit (−366 to 495 bp), including introns of 352 bp and 234 bp in length. B) White and dark bars indicate the location of untranslated and translated sequence sites in the *CGB* prehormone (−20 to 145 aa) and hormone subunit (1–145 aa), respectively. C) The full-length *CGB* amplicons of 548, 423, 300, and 278 bp are shown below the *CGB* gene sequence. aa, amino acid; CTP, CTP of CG6.

endometrial leukocyte proliferation or infiltration (without occasional; some, several; numerous; subepithelial, intraepithelial; massive). Equal or similar endometrial scores for the four features of A–D represent normal secretory-transformed endometrial cycle phases. Decreasing scores for glandular epithelial nuclear transformation, endometrial leukocyte infiltration, or stromal cell density compared with glandular shape configuration indicate insufficient secretory transformation of the endometrium.

Western Blotting

Approximately 50 mg of fresh endometrial tissue samples or tissue stored at −20°C was chopped up, washed, resuspended in ice-cold lysis buffer with the appropriate ingredients, and disrupted under the same conditions as those already described. Clarified endometrial homogenate supernatant was prepared by centrifugation at 19000 × *g* and immediately frozen under the conditions already described. Endometrial protein concentrations were measured in homogenate supernatants using the BCA Protein Assay (Pierce), with values ranging from 2 to 6 mg/ml. Aliquots were diluted in reducing and noneducing probe buffers (Roth) containing SDS and glycerol with or without mercaptoethanol and were boiled at 95°C for 5 min. The prepared endometrial proteins and a low-molecular-weight protein standard mixture and rainbow standard (Pharmacia) were also separated by 10% SDS-PAGE and transferred to nitrocellulose by electroblotting. The resulting membranes were incubated with polyclonal primary rabbit anti-*CGB* (A0231) and rabbit anti-*CGB*-CTP antibodies at a dilution of 1:500 or with monoclonal primary anti-*CGB* (INN-23) and anti-*CGA* (INN-132) antibodies at a dilution of 1:100 at 4°C overnight, followed by incubation with biotinylated secondary goat anti-rabbit (1:2000) or anti-mouse (1:2000) antibodies, as appropriate, for 1 h at room temperature. The membranes were then incubated with ABC complex (Vector). The different molecular forms of hCG were detected by visualization with DAB staining under the same conditions as those described for immunohistochemistry and correlated with the molecular weight markers. Purified dimeric hCG products and *CGB* and *CGA* subunits were obtained from Sigma, Biotrend, and Chemicon, respectively, as positive controls.

RESULTS

To prove that hCG is secreted by normal surface epithelial tissue, we examined human *CGA* and *CGB* mRNA transcription and corresponding hCG dimer protein translation in endometrial samples from patients. Samples ranged from the late proliferative to the late secretory phase of the menstrual cycle. In Figure 1, the *CGB* gene sequence ranging from the transcription start site of −366 bp in the promoter to the transcription stop site at 495 bp in exon 3 is demonstrated for *CGB* gene expression in human placenta [5, 6] and was confirmed for *CGB* gene expression in human secretory endometrial tissue in this study. The resulting *CGB* prehormone (−20 to 145 amino acid [aa]) and *CGB* hormone (1–145 aa) subunit sequences are translated in endometrial secretory tissue. Various *CGB* primer pairs were selected for identification of expressed full-length *CGB* subunits extending from exon 1 to exon 3 (Fig. 1 and Table 1). The *CGA* and *GAPDH* primer pairs were also used.

Evidence for the Presence of hCG in Endometrial Tissue Homogenates and Intrauterine Secretion Material

The total hCG hormone concentrations measured in homogenate supernatants increased from negligible values during the proliferative phase to higher values until the late secretory phase. There was a significant difference between the early secretory phase values and the late secretory phase values ($P < 0.010$). Free *CGB* subunit concentrations corresponded to about one tenth of the dimeric hCG concentration, as evidenced

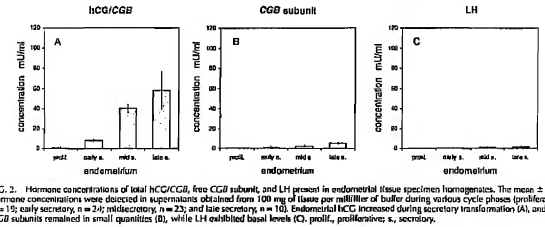

FIG. 2. Hormone concentrations of total hCG/*CGB*, free *CGB* subunit, and LH present in endometrial tissue specimen homogenates. The mean ± SEM hormone concentrations were detected in supernatants obtained from 100 mg of tissue per milliliter of buffer during various cycle phases (proliferative, n = 19; early secretory, n = 24; midsecretory, n = 23; and late secretory, n = 10). Endometrial hCG increased during secretory transformation (A), and free *CGB* subunits remained in small quantities (B), while LH exhibited basal levels (C). prolif., proliferative; s., secretory.

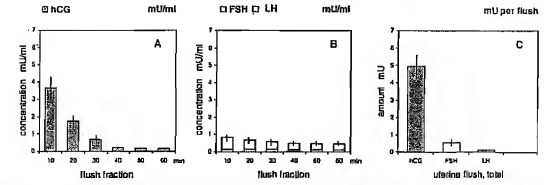

FIG. 3. Uterine flushing in patients during the secretory cycle phase and gonadotropin determination. Uterine flushing was performed using continuous saline infusion and fractionated uterine flushing fluid aliquot collection. Detection of uterine hCG with decreasing sample concentrations (A) and constant basal values for FSH and LH (B) in the successive fluid fractions. (C) The summarized mean ± SEM gonadotropin amounts in the sample fractions are given as milliunits per uterine flush (n = 7).

by the peripheral blood ratio. The LH concentrations tended to be at the low limit of detection and were negligible (Fig. 2). Uterine flushing procedures were performed for seven patients during the midsecretory cycle phase. Fractionated fluid sample collection every 10 min demonstrated a decline in hormone concentrations. The detection limits of the hormone kits were 0.2 mU/ml for hCG, 0.4 mU/ml for FSH, and 0.1 mU/ml for LH. Combining the gonadotropin content from the patients' total uterine flushes resulted in a measurable hormone value for hCG, but LH and FSH remained beyond the limits of detection (Fig. 3). Progesterone and estradiol showed decreasing concentrations in the uterine flush fractions collected, similar to hCG but not prolactin, which revealed constant basal values (data not shown).

Expression of CGB and CGA mRNA in Endometrial Gland Epithelium

Using specific oligonucleotide primer pairs to amplify various exons in the RT-PCR procedure, we obtained the expected *CGB* cDNA amplification products of 548 bp and 423 bp. These results confirmed epithelial *CGB* gene expression in secretory endometrium. Early pregnancy placenta specimens were used as a positive control for hCG expression. Although the already described primer pairs include exon 1 and exon 2 cDNA, additional primer pairs produced 378-bp and 300-bp amplicons containing exon 2 and exon 3, respectively. *CGA* mRNA expression was also evident in secretory endometrium. The absence of *CGA* cDNA when RT was omitted from the assay supported our hypothesis that hCG is expressed throughout endometrial tissue, as indicated by *CGA* and full-length *CGB* mRNA synthesis (Figs. 1 and 4). We semiquantitatively demonstrated that *CGB* sections between exons 1 and 2 (423-bp amplicon) and between exons 2 and 3 (300-bp amplicon) are expressed based on the secretory stage of cyclic endometrium. *CGB* expression began in the early secretory phase and increased to the midsecretory and late secretory phases relative to the amount of constitutive *GAPDH* expression (Fig. 5). To verify the identity of the *CGB* amplicons, DNA cleavage experiments were performed. Digestion of the 300-bp *CGB* amplicon from endometrial and placental tissue with and without various different suitable restriction enzymes (*StyI*, *Bsp*1286, *Hae*III, *AvaII*, and *SmaI*) resulted in the anticipated smaller fragments that characterize the *CGB* DNA origin and prove that *LHB* mRNA was not present (Fig. 6).

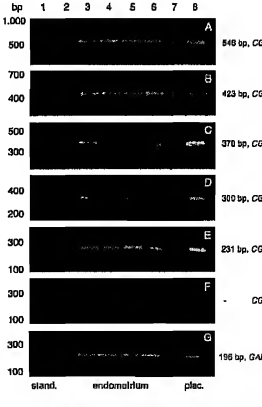

FIG. 4. *CGB* and *CGA* mRNA expression detected during the secretory phase of normal cyclic endometrium and in the placenta control. The RNAs were analyzed by semiquantitative RT-PCR using primer pairs specific to *CGB* (A–D), *CGA* (E and F), and *GAPDH* (G). The panels show the resulting PCR products obtained from various endometrial specimens (lanes 3–6), as well as early gestation placental tissue as a control (lane 8), resulting in *CGB* amplicons of 548 bp (A), 423 bp (B), 378 bp (C), and 300 bp (D). E) *CGA* fragments were also demonstrated in the endometrial tissue specimens (lanes 3–6), in contrast to the reaction procedure performed in the absence of RT activity (F; lane 1: base pair standard; lane 2: without RNA; lane 7: without primer). stand., standard; plac., placenta.

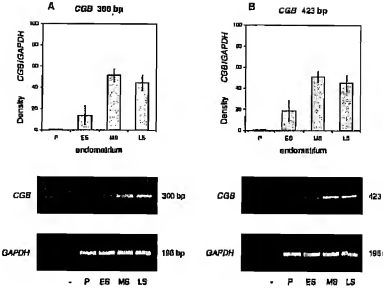

FIG. 5. Dependence of *CGB* mRNA expression on different stages of endometrial secretory transformation. The *CGB* mRNA content of endometrial specimens was determined by RT-PCR relative to the corresponding *GAPDH* amplification for the proliferative (P; $n = 22$), early secretory (ES; $n = 28$), midsecretory (MS; $n = 26$), and late secretory (LS; $n = 15$) phases of the menstrual cycle. The lower gels shows representative amplification products for *CGB* and *GAPDH* using primer pairs amplifying 300-bp (A) and 423-bp (B) products. *CGB* mRNA expression increases in the midsecretory and late secretory endometrial cycle phases. The upper diagrams show the respective optical densitometric ratio of *CGB*:*GAPDH* fragments. Results are given as mean ± SEM.

Production of Endometrial hCG Molecule Forms Evidenced by Western Blotting

To evaluate the detected intrauterine secreted fluid and endometrial homogenate epithelial hCG and to assess the molecular characteristics of endometrial hCG as revealed by immunohistochemistry, we examined the tissue extract fluid of several transformed secretory endometrium specimens and compared it with commercial dimeric and monomeric highly purified commercial placental hCG and early pregnancy serum aliquots. Western blots were performed using different polyclonal and monoclonal *CGB* and *CGA* antibodies and were run under reducing and nonreducing conditions, as shown in Figure 7, A–F. These hCG antibodies were utilized to confirm the molecular hCG pattern using various specific epitope antibody binding possibilities for nonplacental *hCG*. The main bands were approximately 31 and 29 kDa for endometrial *CGB* and 24 and 21 kDa for *CGA*. In addition, numerous hCG dimeric bands of 44, 38, and 35 kDa and CGB subunits of 17 and 15 kDa were detected depending on the desialyzation grade [34–37]. The molecular expression profiles correlate with known molecular isoforms of placental hCG such as those found in serum during pregnancy. Unlike purified placental hCG secretions, endometrial cell homogenate supernatants seemed to contain some additional lower-molecular-weight hCG dimeric and deglycosylated isoforms.

Immunohistochemical Demonstration of hCG Secretion in the Normal Secretory-Transformed Endometrial Epithelium

Endometrial hCG immunostaining of serial tissue sections was performed using polyclonal and monoclonal antibodies recognizing various *CGB* chain epitopes, including the *CTP* sequence. In addition, *CGA*-derived antibody was used to prove total hCG production by human secretory endometrium, as shown in Figure 8. Serial tissue sections of normal cyclic endometrium were evaluated immunohistochemically for hCG during the secretory transformation phase and correlated with cycle-adequate glandular shape transformation, epithelial nuclear differentiation, increasing leukocyte number (CD45), vascular differentiation in endometrial stroma and epithelium (CD34), and endometrial NK cell infiltration (CD56), as shown

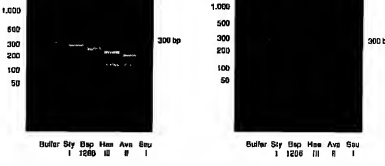

FIG. 6. Effects of restriction enzymes on the cleavage of placental and endometrial *CGB* DNA amplicons. Placental and endometrial RNA was amplified using specific primer pairs for *CGB* to amplify a 300-bp product. A) Placental *CGB* 300-bp amplicon was incubated in buffer without and with enzymes *Sty*I, *Bsp*1286, *Hae*III, *Ava*II, and *Sau*I in lanes 2–7. B) The endometrial *CGB* amplicon was cleaved after enzyme incubations as placental product fragments with *Sty*I (271 bp and 29 bp), *Bsp*1286 (226 bp and 74 bp), *Hae*III (196 bp and 104 bp), *Ava*II (163 bp, 94 bp, 35 bp, and 8 bp), and *Sau*I (154 bp, 94 bp, 35 bp, 9 bp, and 8 bp). The results correlated with the *CGB* DNA sequence but not with the *LHB* sequence.

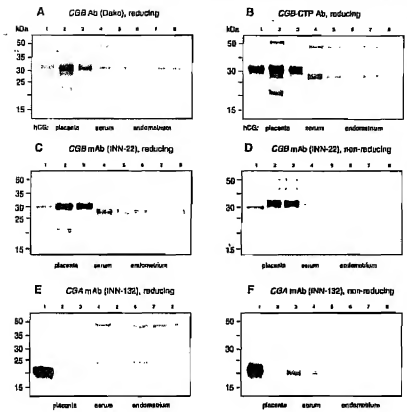

FIG. 7. The SDS-PAGE and Western blotting of hCG present in homogenates of normal secretory endometrium. The Western immunoblot patterns of several endometrial homogenate supernatants (lanes 5–8) were compared with first trimester pregnancy serum (lane 4) and with purified hCG preparations (lanes 1–3). For the latter, hCG from Chemicon (lane 2 in A–D and lane 3 in E and F), hCG from Sigma (lane 3 in A–D and lane 1 in E and F), CGB from Chemicon (lane 1 in A–C), and CGA from Chemicon (lane 3 in E and F) are shown. Molecular weight markers (rainbow and protein standard in kilodaltons) were run together with the described probes. The blots were visualized using polyclonal hCG primary antibody A0231 (A) and polyclonal CGB-CTP primary antibody (B) under reducing conditions, monoclonal CGB primary antibody INN-22 under reducing (C) and nonreducing (D) conditions, and monoclonal CGA primary antibody INN-132 under reducing (E) and nonreducing (F) conditions, followed by biotinylated second antibody, ABC complex, and DAB staining. Ab, antibody; mAb, monoclonal antibody.

in Figure 9. The immunolocalization indicated that glandular epithelial cells are the only site of uterine hCG expression. Beginning with secretion in the developing epithelial subnuclear vacuoles, hCG became more prominent during the higher phases of secretory transformation. Normal synchronous secretory transformation is confirmed by the following observed degrees (range, 0–4) of immunohistochemical hCG staining in Figure 9: approximately 0.5–1 for very early (Fig. 9B), 2 for early secretory (Fig. 9C), 3 for midsecretory (Fig. 9D), 3–4 for late secretory (Fig. 9E), and 4 for predecidual late secretory (Fig. 9F) cycle endometrial sections. This also correlates with assessed endometrial tissue features (glandular shape configuration, glandular nucleus configuration, stroma configuration, and leukocyte infiltration/proliferation). Figure 9 also shows normal cyclic endometrium with synchronized tissue features and endometrial scores (range, 1–6) of 1–2 for proliferative, 2–3 for early secretory, 3–4 for midsecretory, and 5–6 for late and predecidualized late secretory endometrium for each feature. Thus, a total endometrial score exceeding 20 could be achieved for the four tissue features in normal late secretory transformation. The large amount of mononuclear cells and vascularization present in endometrial tissue with high hCG expression could be the result of chemoattraction and improved vascularization described for hCG [38]. Distinct subepithelial and, especially, intraepithelial vascularization is shown in Figures 9 and 11. We assume that epithelial hCG present during the high secretory cycle phase not only is sent to the glandular lumen for secretion but also is targeted to the peripheral blood vessels. Epithelial hCG secretion has been found in apical glandular epithelium of secretory-transformed epithelium but is also accompanied by secretion in luminal surface epithelium during the menstrual cycle (Fig. 10).

Failing hCG Production as a Criterion for Abnormal Endometrial Secretory Differentiation

Endometrial hCG production in glandular epithelium correlated with synchronous transformation of glandular shape configuration, glandular epithelial nucleus configuration, and/or leukocyte infiltration into normal secretory endometrium (Figs. 9 and 10). Numerous suboptimal or disturbed secretory endometrial specimens were found in association with infertility diagnoses in this study. In these cases, a delay in endometrial maturation of longer than 2 days (evidenced by inadequate progesterone secretion) is thought to result in disturbed implantation and in early abortion. Criteria for histological assessment of endometrium biopsy specimens are routinely used to characterize normal or delayed secretory transformation and the accompanying postovulatory cycle day [27]. Similarly, glandular-stromal dyssynchrony has been described as a cause of endometrial dysfunction [39]. In patients with diminished or absent epithelial hCG staining, we observed dyssynchronous endometrial differentiation, especially in glandular nuclear transformation and/or endometrial leukocyte infiltration to the glandular shape configuration (Fig. 11). Surprisingly, disturbed endometrial secretory differentiation can be recognized by a single immunohistochemical hCG staining of endometrial biopsy material obtained for infertility diagnosis. The following thresholds of decreasing endometrial tissue scores (range, 1–6), compared with an average score of

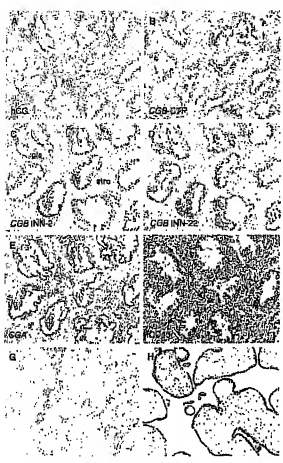

FIG. 8. Immunohistochemical staining using different *CGB* and *CGA* antibodies for visualization of endometrial hCG production. Evidence of hCG formation in secretory glandular epithelium of serial slides indicated by polyclonal antibody A0231 (A), polyclonal antibody hCG-CTP from Biotrend (B), monoclonal antibody INN-2 (C), and monoclonal antibody INN-22 (D), all for *CGB*, as well as by monoclonal antibody INN-132 (E) for *CGA* and hematoxylin staining together with a leukocyte CD45 antibody (F). Negative (G) and positive (H) controls for hCG staining are shown. glu, glandular epithel; stro, stromal cells; leu, leukocytes. Original magnification ×200.

4.5 for cycle-adequate normal glandular shape configuration, were associated with missing synchrony: 3 for stromal cell density, 2.5 for glandular nuclear shape configuration, and 2 for endometrial leukocyte infiltration (Fig. 11). These characteristics are associated with insufficient normal secretory transformation of the endometrium.

DISCUSSION

The objective of our study was to prove that normal secretory endometrium is capable of producing both transcription and translation hCG subunits during the healthy menstrual cycle. The results herein demonstrate that the endometrium of women primarily during the secretory phase expresses, produces, and secretes dimeric hCG hormone in glandular and luminal epithelium. This endometrial hCG is characterized by *CGB* and *CGA* subunit sequence expression [5–7]. Human chorionic gonadotropin was shown to be present by measuring hormone levels in uterine lavage fluids and endometrial homogenates. Restriction enzyme examination excluded *LHB* expression. Various molecular forms of the *CGB* and *CGA* subunits (such as dimeric hCG) were also observed for endometrial tissue, which demonstrates a molecular pattern similar to that exhibited by placental hCG [34–37]. Human chorionic gonadotropin is expressed and released maximally in secretory-transformed endometrium with the highest evaluated scores. Increasing hCG production and greater quantities of endometrial mononuclear cells (such as common leukocytes [CD45] and NK cells [CD56]) correlate with cycle phase-adequate secretory transformation during the normal healthy menstrual cycle. Endometrial hCG in glandular epithelium is secreted in cavum uteri.

The effects of progesterone, along with the resulting receptor expression, have been considered the main cause of endometrial secretory transformation [1, 40]. It is generally assumed that only the additional influence of fetal hCG induces decidual transformation in progesterone-conditioned and secretory-transformed cyclic endometrium. It has been well studied that exogenous hCG administration acts like fetal hCG in having an immediate influence on predecidual function of human cyclic endometrium [3, 41, 42, 43]. The effect of hCG on human secretory endometrium promotes morphological and functional differentiation of stromal cells into decidual cells [41, 42]. However, even in the absence of implantation, predecidual reactions of stromal fibroblast cell transformation leading to epithelial gland cell hyperplasia (including plaque formation in the luminal epithelium) occur in human secretory endometrium, while this predecidual cell transformation is absent during the normal estrogen- and progesterone-primed menstrual cycle of nonhuman primates [4, 44, 45]. Subsequent exogenous hCG administration also induces predecidual transformation.

The effect of midcyclic hCG administration was investigated in a patient group having normal menstrual cycles in which transformation of secretory endometrium was examined [43]. Compared with biopsy specimens lacking hCG administration, biopsy specimens of hCG-primed endometrium demonstrated reinforced development of glandular dilatation, progressive nuclear differentiation in epithelial cells, decreased epithelial and stromal cell mitosis rates, and increasing numbers of spiral arteries. Therefore, our immunohistochemical hCG staining results for patients with normal secretory endometrium suggest that exogenous hCG, and possibly endogenous hCG released in glandular epithelium, induces secretory transformation and predecidualization of nonpregnant endometrium.

We believe that endogenous endometrial hCG is responsible for predecidual development of secretory endometrium in women. Similar to the effect of exogenous hCG on secretory transformation of the endometrium as described by other authors [46], the menstrual cycle-dependent effect of endogenous hCG on human endometrium is confirmed herein. We hypothesize that endogenous hCG released by glandular epithelium can induce and control glandular and stromal endometrial differentiation in a paracrine fashion and/or an endocrine fashion. As evidenced by immunohistochemical staining results, our findings suggest that endometrial hCG may contribute, as early as the early secretory phase, to glandular development, along with increased endometrial vascularization, stromal differentiation, and proliferation or infiltration of endometrial mononuclear cells. Adequate glandular hCG secretion potentially optimizes secretory differentiation of fertile cyclic endometrium and prepares the tissue for embryo implantation and successful gestation. During the early

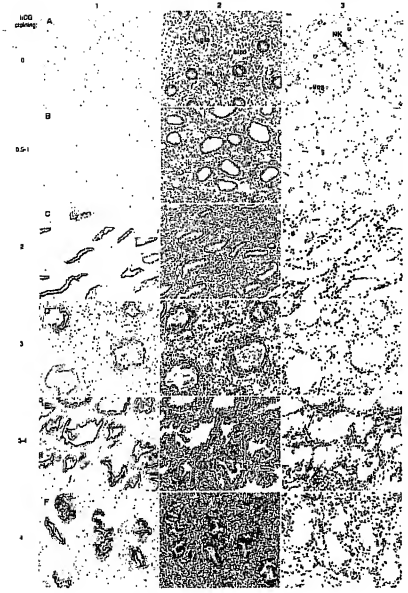

FIG. 9. Immunohistochemical demonstration of endometrial hCG secretion in glandular epithelium; hCG staining is given as the degree of staining (range, 0–4). Lane 1: Lack of hCG production in the endometrial proliferative phase (A), beginning of hCG secretion in subnuclear glandular vacuoles (B); intensified and increasing glandular hCG secretion during the early secretory (C) and midsecretory (D) phases, respectively; and strong glandular hCG secretion in functionalis (E) and predecidual (F) endometrium areas of the late secretory phase. Human chorionic gonadotropin production characterized by an hCG staining degree of 0–4 correlates with adequate glandular shape transformation, epithelial nuclear differentiation, and leukocyte development suitable for normal secretory endometrial function of the menstrual cycle (lane 2: hematoxylin staining and monoclonal antibody CD45 mononuclear immune cells). Endometrial hCG secretion refers to the proliferation of endometrial NK cells and the vascular differentiation of endometrial stroma (lane 3: monoclonal antibody CD56 for NK cells and monoclonal antibody CD34 for endothelial cells). gla, glandular epithel; stro, stromal cells; leu, leukocytes; NK, endometrial NK cells; vas, vessels. Original magnification ×200.

secretory to late secretory phases, epithelial hCG is thought to stimulate immunologically detected increasing gland-surrounded subepithelial and, above all, periepithelial vascularization in endometrial specimens, similar to exogenously administered hCG [47]. Like exogenous hCG, increased production of endometrial hCG may support endometrial predecidualization of stromal fibroblast induction of vascular endothelial growth factor (VEGF), IGFBP1, and αSMA, as well as the resulting formation of epithelial plaques [41, 44].

Because hCG expression correlates with progesterone stimulus, we hypothesized that hCG formation is a progesterone-induced process. Furthermore, because hCG is regarded as an immunoregulatory hormone, it could have an important role during implantation. Therefore, we speculated that endometrial hCG may be an appropriate marker to assess the receptivity of the endometrium for embryo implantation. Increasing hCG production correlates with endometrial secretory transformation in the normal healthy menstrual cycle, characterized by phase-synchronous differentiation with regard to gland size, glandular nuclear transformation, stromal cell density, and stromal mononuclear cell number or subepithelial and periepithelial vessel density in the endometrium. Thus, we recommend performing immunohistochemical hCG detection in the endometrium as a diagnostic method to evaluate the degree of endometrial secretory transformation (as assessed by degree of immunohistochemical hCG staining [range, 0–4]). We also provide evidence for dyssynchronous endometrial differentiation in which gland nuclear transformation is delayed

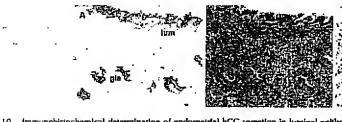

FIG. 10. Immunohistochemical determination of endometrial hCG secretion in luminal epithelium. A) Epithelial hCG is produced in apical glandular and luminal surface epithelium during the secretory cycle phase, shown here in the late secretory predecidual epithelial cluster region. B) Massive numbers of leukocytes infiltrate the stromal area. C) Subepithelial NK cells are visible in glandular and luminal epithelium, as well as intense vessel formation surrounding epithelium. gla, glandular epithel; lum, luminal surface epithel; leu, leukocytes; NK, endometrial NK cells; ves, vessels. Original magnification ×200.

with respect to phase-adequate gland size and in which the stromal leukocyte number is diminished in patients with insufficient endometrial secretory transformation. In such patients, glandular hCG formation is decreased or absent on immunohistologic examination. The proposed morphological assessment confirms the results of immunohistochemical evaluation of endometrial hCG staining. In general, the eight criteria by Noyes et al. [27] for histological assessment of endometrium biopsy specimens are used to characterize secretory transformation and accompanying postovulatory cycle day. These morphologically objective and diagnostically subjective criteria are insufficiently defined for the characterization of endometrium development [32], but their application is still recommended for assessment of inadequate effects of progesterone and for exclusion of endometrial anomalies if no modalities are available for recording of endometrial maturation [33]. We prefer validation of endometrium biopsy specimens and, above all, use of the proposed hCG immunohistochemical evaluation for validation of cycle phase-synchronous or dyssynchronous secretory-transformed endometrium, as well as the four parameters described herein (glandular shape configuration, glandular epithelial nucleus transformation and localization, stromal cell density and configuration, and endometrial leukocyte proliferation and infiltration).

Endometrial expression of the hCG/LH receptor is subject to cyclic endometrial variations and increases concomitantly with progress of the secretory phase [22, 23]. Epithelial cells of the fallopian tube also express hCG/LH receptor and secrete hCG [21, 24]. Functional full-length hCG/LH receptor is downregulated in the endometrium during the late secretory cycle stage and in early gestation decidua [48]. Increasing endogenous hCG concentrations in normal secretory endometrium may influence receptor downregulation.

We showed in this study that local epithelial hCG synthesis by healthy secretory endometrium, in association with intensive subepithelial vascularization and stromal NK cell accumulation, lasts until the late secretory phase, including its predecidually induced hypertrophic epithelial cell areas. Numerous in vitro and in vivo investigations have examined the influence of exogenous hCG doses on morphological and functional differentiation of endometrial tissues, including phenomena such as apoptosis. Untreated control cycles in these studies resulted during the late secretory phase predominantly in ischemic necrotic stromal areas of the endometrium before menstruation [49, 50]. In these untreated cycles, despite a declining BCL2:BAX ratio that leads merely to minimal cellular apoptosis during the late secretory phase, the number of apoptotic cells present in the premenstrual endometrial glandular epithelium and stromal cells amounts to less than 2% [51, 52]. Comparative endometrial biopsy specimens from initially untreated and subsequently hCG-treated patient cycles show strongly decreased apoptosis after therapy [53]. This indicates that hCG suppresses cellular apoptosis in endometrial gland epithelial and stroma cells and explains the absence of apoptosis in decidual epithelium during early gestation associated with high hCG concentrations [54]. Therefore, our observation of glandular hCG in midsecretory and late secretory endometrium supports the findings of a low ratio of apoptosis during the normal menstrual cycle and at the time of menses, as well as the presence of glandular hCG in decidua during early gestation [55].

With every menstrual cycle, angiogenesis is repeatedly stimulated in the developing endometrium in a manner that does not occur in other organs. During this time, endometrial vascularization is of fundamental importance for generation of a receptive endometrium. Endometrial angiogenesis is induced predominantly by increasing VEGF expression in epithelial and stromal cells during midsecretory to late secretory cycle stages [56–58]. Expressed VEGF family proteins achieve optimum values only in the late secretory cycle phase, when endothelial cell proliferation and endometrial predecidualization are high, and are released in small quantities. In addition, endometrial angiogenesis and VEGF expression can be stimulated by embryo-derived hCG during implantation and ongoing pregnancy. Endometrial cell cultures and intrauterine microdialysis measurements confirm the indirect influence of hCG on vascularization by increased VEGF expression in epithelial and stromal cells [59, 60]. In contrast, hCG can directly trigger angiogenic activities of endometrial hCG/LH receptor interaction for increased capillary function. Various in vivo and in vitro methods such as endothelial cell proliferation, microvessel sprouting, and vessel density measurements confirm the initiation of angiogenesis in response to hCG binding of the endothelial hCG/LH receptor [47, 61].

To ensure adequate uterine angiogenesis until the time of implantation and development of decidual circulation, endometrial angiogenesis was evaluated together with the effect of embryonic hCG on endometrial hCG/LH receptors as the key to vascular changes in endometrium and decidua and in decidual angiogenesis. We believe that the results herein prove that physiological, modest, endogenous endometrial hCG release from glandular epithelium can have a direct or an indirect role in the effect of hCG on the hCG/LH receptor or on VEGF regulation at the beginning of the early secretory cycle phase and can contribute to the initiation of microvascularization in preparation for a receptive endometrium. The immunohistochemical results herein show increased subepithelial and intraepithelial vessel growth development, which correlates with the strength of hCG expression in glandular

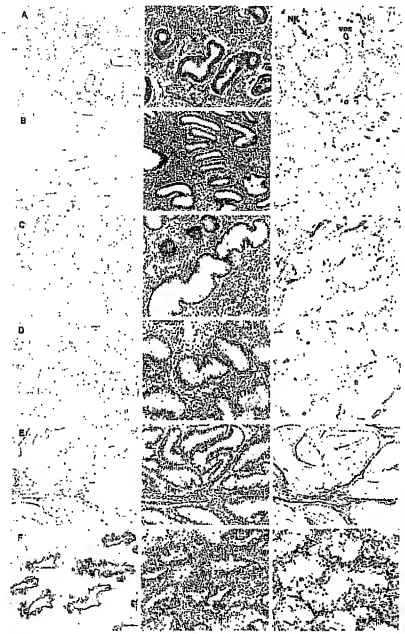

FIG. 11. Immunohistochemical hCG staining to evaluate insufficient secretory differentiation of the endometrium. Representative findings of suboptimal endometrial transformation are shown for serial slides of dyssynchronous or failing secretory endometrium stained for hCG (left), mononuclear cells CD45/hematoxylin (middle), and NK cells CD56/vessel endothelium CD34 (right). Adequate midsecretory and late secretory phase endometrial glands show small quantities or absence of hCG, with diminished numbers of stromal leukocytes and inadequate palisadification (A), phase inadequate appearance of subnuclear vacuoles (B), and cylinder-shaped epithelial cell nuclear differentiation (C). Endometrial hCG formation also fails to occur in the absence of stromal mononuclear cells, despite normal epithelial nuclear differentiation (D) and an abnormally increased ratio of glands/stroma area (E). Positive controls for staining are included (F). glo, glandular epithelial slro, stromal cells; leu, leukocytes; NK, endometrial NK cells; ves, vessels. Original magnification ×200.

epithelium of nonpregnant endometrium during the midsecretory to late secretory and predecidual secretory cycle stages. The results indicate that endometrial hCG is delivered to the uterine cavum and possibly to the venous reflux of the uterus and directly into the circulation. Physiologically, hCG is found in the peripheral blood of healthy nonpregnant fertile women, and the level can reach up to 3.0 mU/ml (higher than that in men). The free *CGB* subunit concentration is considerably lower than that of corresponding dimeric hCG [20]. We confirm much higher dimeric hCG concentrations compared with free *CGB* subunit concentrations in our results showing hCG release into the homogenate supernatant of high secretory cycle phase endometrium specimens, as well as in our premenstrual peripheral blood measurements of hCG in patient studies (data not shown). In the epithelium of various tissues, the free *CGB* subunit can be exclusively expressed and released, or a functional dimeric hCG may be produced in small quantities [16, 62].

Human chorionic gonadotropin is thought to exert an immunomodulating effect on the epithelial surface. In

endometrial glandular epithelium and stroma of the secretory cycle phase, Fas ligand (FASL) and Fas receptor (FAS) are increasingly coexpressed, which results in autocrine epithelial apoptosis only during the late secretory phase characterized by decreasing estrogen and progesterone concentrations [63–65]. General FASL expression in epithelial cells, as shown in endometrium, was described for the first time in corneal epithelium of the eye, in testicles, and in placenta, creating immune-privileged sites for these tissue areas via apoptosis induction and defense of allogeneic Fas-positive immune cells (lymphocytes) and inhibiting immunological inflammatory reactions [66–68]. The observation of endogenous hCG production in secretory endometrium herein seems important in light of the recent findings that hCG alone can cause strong estrogen-independent and progesterone-independent induction of endometrial FASL expression in glandular epithelium and stroma [69]. Therefore, the observed endogenous hCG likely supports FASL expression in secretory endometrium in a cycle-dependent manner. The finding of endogenous hCG in the endometrium may also correlate with FASL expression in epithelial and stroma cells, resulting in an immune-privileged site capable of repulsing Fas-bearing lymphoid cells such as activated T lymphocytes. Besides the endometrium, eye, testis, and placenta, other sites of epithelial FASL expression have been found in normal human tissues such as lung, esophagus, and prostate [70]. Numerous epithelial FASL production sites are associated with exactly the same expression of epithelial hCG. If the concept of an immune-privileged site is extended to FASL and hCG coexpression in epithelia, the epithelial surfaces of respiratory, enteric, and urogenital tracts could be included to a large extent in characterizing the extrinsic defense space of induced apoptosis of inflammatory cells, infectious bacteria, or allogeneic grafts without risky immunological reactions in tissues. Furthermore, hCG secreted by early trophoblast or maternal decidua attracts regulatory T cells to the fetomaternal interface and can contribute to maternal tolerance toward the fetus [55, 71].

In conclusion, this study demonstrates for the first time (to our knowledge) that *CGB* and *CGA* subunits and hCG are expressed and produced in glandular epithelium of synchronous progesterone-stimulated secretory endometrium. Maximal hCG production correlated with maximal mononuclear cell occurrence and with considerable vascularization. Therefore, we believe that endometrial hCG is a marker for receptivity of embryo implantation, although further investigation is required.

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